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Original article

Deletion of *PdMit1*, a homolog of yeast *Csg1*, affects growth and Ca^{2+} sensitivity of the fungus *Penicillium digitatum*, but does not alter virulence

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Abstract

GDP-mannose:inositol-phosphorylceramide (MIPC) and its derivatives are important for Ca^{2+} sensitization of *Saccharomyces cerevisiae* and for the virulence of *Candida albicans*, but its role in the virulence of plant fungal pathogens remains unclear. In this study, we report the identification and functional characterization of *PdMit1*, the gene encoding MIPC synthase in *Penicillium digitatum*, one of the most important pathogens of postharvest citrus fruits. To understand the function of *PdMit1*, a *PdMit1* deletion mutant was generated. Compared to its wild-type control, the *PdMit1* deletion mutant exhibited slow radial growth, decreased conidia production and delayed conidial germination, suggesting that *PdMit1* is important for the growth of mycelium, sporulation and conidial germination. The *PdMit1* deletion mutant also showed hypersensitivity to Ca^{2+}. Treatment with 250 mmol/l Ca^{2+} induced vacuole fusion in the wild-type strain, but not in the *PdMit1* deletion mutant. Treatment with 250 mmol/l$CaCl_2$ upregulated three Ca^{2+}-ATPase genes in the wild-type strain, and this was significantly inhibited in the *PdMit1* deletion mutant. These results suggest that *PdMit1* may have a role in regulating vacuole fusion and expression of Ca^{2+}-ATPase genes by controlling biosynthesis of MIPC, and thereby imparts *P. digitatum* Ca^{2+} tolerance. However, we found that *PdMit1* is dispensable for virulence of *P. digitatum*.

Keywords: *Penicillium digitatum*; GDP-mannose:inositol-phosphorylceramide; *PdMit1*; Ca^{2+} sensitivity; Ca^{2+}-ATPase

1. Introduction

Sphingolipids are ubiquitous lipid components of eukaryotes, including filamentous fungi. In addition to the structural components of membranes, sphingolipids and their metabolites are now recognized as important bioactive molecules that modulate various cellular events, including cell proliferation, differentiation, endocytosis, apoptosis, nutrient uptake, calcium homeostasis and the stress response [1,2]. Fungal sphingolipids are distinct from those found in plants, animals and humans, and the enzymes responsible for synthesis of fungus-specific sphingolipids may serve as potential targets of antifungal drugs [3,4].

Biosynthesis of sphingolipids has been intensively studied in *Saccharomyces cerevisiae* [5,6]. It starts with condensation of palmitoyl-CoA and serine in the endoplasmic reticulum [7]. After undergoing a series of reactions, including reduction, hydroxylation and acylation, phytoceramide is synthesized [8]. Thereafter, IPC synthase encoded by *Aur1* transfers a phosphoryl-inositol moiety from phosphatidylinositol to the 1-hydroxy group of phytoceramide to form IPC (inositol-phosphorylceramide) [9]. IPC is further

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modified by the addition of mannose to form MIPC (mannose inositol-phosphorylceramide), and then another inositol phosphate group is further introduced to MIPC by inositol phosphotransferase to form $M(IP)_2C$. In the plasma membrane, IPC, MIPC, $M(IP)_2C$ and other complex sphingolipids constitute the lipid rafts with cholesterol and sterols [10]. Lipid rafts have been elaborated to participate in assembly of the subcortical actin cytoskeleton and are involved in material transport and endocytosis [11,12]. Two distinct MIPC synthase complexes, *Csg1* (calcium sensitive gene)-*Csg2* and *Csh1* (*Csg1* homolog)-*Csg2*, participate in the addition of mannose to the inositol 2-OH moiety of IPC to form MIPC in *S. cerevisiae*. The subunits encoded by *Csg1* and *Csh1* perform catalysis and the subunit encoded by *Csg2* performs a regulatory function [13].

In *S. cerevisiae*, the absence of both *Csg1* and *Csg2* resulted in a significant reduction in MIPC. Accumulation of IPC-C was also observed in this double gene deletion mutant [14]. It was reported that *Csg1* and *Csg2* play an important role in Ca^{2+} tolerance in *S. cerevisiae*. Moderate concentrations (about 100 mmol/l) of Ca^{2+} could induce death of the *Csg1* and *Csg2* double deletion mutant [15]. A *Csg1 homolog* deletion mutant of *Candida albicans*, *Aspergillus fumigates* and *Schizosaccharomyce pombe* also exhibited increased Ca^{2+} sensitivity [14,16–18]. When accumulated IPC-C was converted into IPC-D, the precursor of MIPC, Ca^{2+} sensitivity of the MIPC synthase-deficient mutant was suppressed, suggesting that the accumulation of IPC-C is associated with Ca^{2+} sensitivity [15,18].

MitA in *Aspergillus fumigatus* was the first *Csg1* homolog to be characterized in filamentous fungi. In the *MitA* deletion mutant of *A. fumigatus*, IPCs accumulated, whereas $M(IP)_2C$ was absent due to a lack of transfer of mannose to IPCs [18]. Conidia germination of the *MitA* deletion mutant of *A. fumigatus* was decelerated under normal growth conditions, but the virulence of this mutant was not impaired [18].

Mit1, the homolog of *S. cerevisiae Csg1*, was also required for formation of MIPC-derived complex sphingolipids and phospholipomannan in *C. albicans* [17]. A previous study found that β-mannosylation of phospholipomannan played a role in the virulence of *C. albicans* by contacting host cells and diffusing into the cell wall [19]. Consistently, the *Mit1* deletion mutant of *C. albicans* was less virulent during both the acute and chronic phases of systemic infection in mice due to reduced β-mannosylation of phospholipomannan [17].

Citrus green mold caused by *Penicillium digitatum* is the most destructive disease of postharvest citrus fruits and causes significant loss during post-harvest storing, packaging, transportation and marketing of citrus fruits [20]. Despite its economic importance, the molecular mechanisms involved in growth, sporulation, adaptation to the changing environment and pathogenesis of *P. digitatum* have not been fully elucidated. In this study, we report the identification of *PdMit1*, an ortholog of *Csg1*/*Mit1* from *P. digitatum*, and we demonstrate that *PdMit1* is important for mycelial growth, sporulation and conidial germination of *P. digitatum*, but not for virulence.

2. Materials and methods

2.1. Fungal strains and culture conditions

Wild-type strain PdKH8 of *P. digitatum* used in this study was collected from Kaihua County, Zhejiang Province, China in 2010. Both the wild-type strain and its derivative mutant strains were cultured on PDA (potato dextrose agar) at 25 °C. Conidia were obtained by scraping the agar surface with sterile distilled water at 5 dpi (days post inoculation).

2.2. Sequence analysis of PdMit1

To identify the putative gene encoding MIPC synthase in *P. digitatum*, the amino acid sequences of *S. cerevisiae* Csg1p/Sur1p (systematic name, YPL057C), Csh1p (YBR161W) and Csg2p (YBR036C) [15,21] were used as queries in BLASTp search of the *P. digitatum* genome [22]. Sequences of DNA and cDNA of *PdMit1* were amplified from the genomic DNA and cDNA of wild-type strain PdKH8, respectively, with primers Mit1-F/Mit1-R (Table S1). The amplified fragments were cloned into the pMD18-T vector and sequenced. The putative protein of *PdMit1*p was predicted for subcellular localization using TargetP 1.1 (www.cbs.dtu.dk/services/TargetP/). SignalP 4.1 (www.cbs.dtu.dk/services/SignalP/) and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) were used for the analysis of transmembrane domains (TMDs) and the location of signal peptide cleavage sites, respectively.

2.3. Construction of the PdMit1 deletion mutant

Two flanking sequences of the *PdMit1* gene amplified from *P. digitatum* genomic DNA were inserted upstream and downstream of the *hph* (hygromycin resistance) gene of the pTFCM vector, respectively. A 1439 bp fragment upstream of the *PdMit1* coding sequence was amplified with Mit1-up-F and Mit1-up-R (Table S1) and cloned into the *Kpn*I and *Sac*I sites of pTFCM vector to construct PTFCM-*PdMit1*-up. Then, a 1432 bp fragment downstream of the *PdMit1* coding sequence was amplified using primers Mit1-down-F and Mit1-down-R (Table S1) and inserted into the *Spe*I and *Xho*I sites of the pTFCM-*PdMit1*-up vector to generate *PdMit1* replacement vector pTFCM-△*PdMit1*. This replacement vector was transformed into *Agrobacterium tumefaciens* AGL-1 by electroporation using ECM630 (BTX, CA, USA). To obtain the transformants, *A. tumefaciens*-mediated transformation (ATMT) was performed as described previously [23]. The *PdMit1* deletion mutants were selected on PDA medium supplemented with 70 μg/ml hygromycin B. The identification of gene replacement mutants was performed by PCR using primers Mit1–check-F1/Mit1–check-R1 and Mit1–check-F2/Mit1–check-R2 (Table S1). Southern hybridization analysis was used to further confirm the *PdMit1* replacement. Genomic DNA of the *PdMit1* deletion mutant or wild-type strain was cut with the *EcoR*II and *BamH*I restriction enzymes, respectively. The probe (787 bp) corresponding to the upstream flanking sequence of the *PdMit1* gene was amplified